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Effects of HIV-1 Nef on Cellular Gene Expression Profiles

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Abstract
The early human immunodeficiency virus (HIV) accessory protein Nef makes an important contribution to virulence, but the mechanisms by which Nef influences pathogenesis remain unclear. Many well-studied effects of Nef, like CD4 and class I MHC downregulation, occur posttranslationally. However, Nef has the potential to affect gene expression by interfering with cell signaling pathways and by virtue of structural features such as the Pro-X-X-Pro motif, which may interact with *src* homology region-3 domains of src-like kinases. We used a cDNA microarray screening strategy to identify cellular genes whose steady state transcriptional levels may be affected by Nef. We generated HeLa cell lines expressing wild-type or mutant HIV-1 *nef* protein sequences. Using cDNA microarray technology, we compared the patterns of cellular gene expression in the various cell lines to the pattern in non-Nef-expressing HeLa cells. By matching the patterns of cellular gene expression in HeLa cell lines expressing various Nefs with that of parental HeLa cells, we identified several cellular genes whose expression was modulated differentially by Nef and its mutants. We confirmed the differential expression of selected genes by RNA filter blotting. Genes expressed at higher levels included proteases, transcription factors, protein kinases, nuclear import/export proteins, adaptor molecules and cyclins, some of which have previously been implicated as being important for HIV replication and pathogenesis. The results indicate that Nef expression can alter the expression of cellular genes and suggest that this alteration in cellular gene expression may serve to optimize the cell to support the subsequent stages of viral replication.

Introduction

Human immunodeficiency virus type 1 (HIV-1) Nef is a phosphorylated, N-myristylated protein expressed early in the viral replication cycle. The protein is highly conserved across viral strains and is an important virulence factor. Virus containing *nef* mutations does not replicate as well as the wild type (wt), and *nef*-defective virions are approximately 5–10 times less infectious than wt HIV-1 when tested in a single-cycle infection assay [25, 75, 89]. In vivo, *nef* is required for high-level virus replication

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[58]. Although *nef* clearly has an important impact on pathogenesis, the mechanisms through which *nef* contributes to pathogenesis remain unclear.

Nef appears to have multiple functions. Nef can (1) enhance viral replication by promoting an increased rate of viral cDNA synthesis [24, 89], (2) downregulate cell surface CD4, perhaps improving viral release [1, 42], (3) downregulate class I MHC (MHC-I) molecules, probably protecting infected cells against killing by cytotoxic T lymphocytes [14, 28, 41, 90] and (4) affect cell signaling pathways in several cell types [35], likely by associating with a cellular serine kinase [10, 27, 46, 54, 69] and by disabling the inositol triphosphate receptor [45].

Nef is predominantly localized in the cytoplasm and is associated with the inner leaflet of the plasma membrane; mutation of glycine at position 2 prevents myristylation and precludes association of the mutant protein with the cell membrane [78, 110], thereby preventing its interaction with signaling pathways. Plasma membrane-associated Nef is incorporated into virions and is processed within virions by the viral protease [80]. Within virions, Nef is associated with the viral core [64]. A 2-leucine motif within the N-terminal region of Nef is critical for CD4 downregulation [1]. The region between amino acids 69 and 80 of HIV-1 Nef contains two repeats of the sequence Pro-X-X-Pro (PXXP), a motif that mediates the interactions of the *src* homology region-3 (SH3) domains of several cellular signal transduction proteins.

The effects of Nef on cell signaling may have particular pathogenic significance. In T cell lines, Nef blocks an early event in CD3-initiated signaling and early gene expression [10]. The mechanisms through which Nef alters cell signaling pathways may involve the SH3-like PXXP domain. In virus-infected lymphoid cells, Nef interacts with a cellular serine/threonine kinase, designated Nef-associated kinase (NAK) [59]. Recently, NAK has been identified as cellular p21-activated kinase [38]. A number of other cell signaling proteins, including tyrosine (Lck, Hck, Src and Lyn) and serine/threonine kinase (protein kinase C-theta), have been reported to associate with Nef [86]. The interaction with kinases may contribute to HIV pathogenesis, since the ability of Nef to associate with NAK was correlated with progression to fatal simian AIDS [59]. The interactions of Nef with components of the signal transduction machinery could be important for the replication of HIV and SIV (Simian Immunodeficiency Virus) in primary cells, particularly in resting or suboptimally stimulated T cells [35, 72, 75, 86, 97].

Some Nef effects, such as CD4 and MHC-1 downregulation, result from posttranscriptional effects. However, the structural features of Nef and its demonstrated ability to interact with host cell signaling pathways suggest that at least some of its activities involve alterations in host cell gene expression. Given the many potential mechanisms of action and the relatively small amount of information available concerning the effects of Nef on the host cell, the detailed pathways mediating the effects of Nef are not obvious. We therefore conducted studies to broadly characterize the effects of Nef on host cell gene expression. We used a series of HeLa cell lines expressing wt NL4-3 Nef or mutants with changes in certain domains such as the myristylation site, nuclear localization domain and PXXP motif. Using cDNA microarray technology, which provides quantitative information on the relative expression levels of many genes simultaneously [88], we compared the transcriptional patterns in HeLa cells expressing wt- or mutant Nef with that of control HeLa cells. We found that Nef alters the expression of a limited but significant subset of cellular genes when expressed in HeLa cells. These differentially regulated genes constitute a diverse assortment of genes, including genes involved in transcription, intracellular metabolism, cell cycle regulation, signal transduction, RNA metabolism, nuclear-cytoplasmic trafficking, DNA mismatch repair, vesicle/membrane fusion and the cytoskeleton. Among these are some genes that have previously been identified as being involved in HIV replication. The data suggest that although *nef* is not essential for viral replication, it serves to enhance the ability of the host cell to support HIV replication in a variety of ways. The results of this study indicate that Nef may have profound effects on viral infection at a transcriptional level and that its effects are linked to certain structural determinants in the Nef protein sequence. The results may suggest additional targets for antiviral drug design and may offer deeper insights into Nef function.

Methods

Plasmids and Cells

Various Nef alleles were PCR amplified from the respective proviral DNAs, with six histidine tags at the C-termini, and cloned into pCR3.1 vector (Invitrogen). The Nef mutants described in this study were engineered into the NL4-3 Nef by the Mutagene technique (Bio-Rad Corp.). In the PXXP→AXXA mutant, the sequence -PVT*P*QV*P*LRP- between residues 69 and 78 was mutated to -PVT*A*QV*A*LRP-, abolishing all three -PXXP- motifs in this region. The various Nef inserts from the respective pCR3.1 recombinants were verified by sequencing and cloned as *Eco*RI fragments into the bacterial expression vector, pCR2.1. Nef expression was verified by Western blotting (with a cocktail of anti-Nef monoclonal antibodies

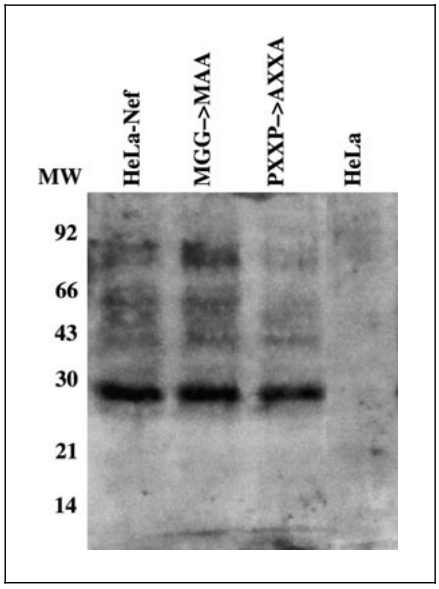

Fig. 1. Immunoblotting of extracts from HeLa cells expressing wt- and mutant Nefs. Extracts were produced from HeLa cells stably transfected with a vector expressing wt-Nef (NL4-3), Nef mutated in the myristylation site (MGG→MAA) or Nef mutated in the SH3 domain (PXXP→AXXA). Extracts were subjected to SDS-PAGE and immunoblotted. The wt and mutant cell lines express similar amounts of protein. MW = Molecular weight.

or rabbit anti-Nef sera). Also, SDS-PAGE analysis of bacterial extracts prepared after IPTG induction of pCR2.1 recombinants was carried out to determine protein expression levels (data not shown).

HeLa cells were transfected with individual Nef recombinants and clones selected on G418 (1 mg/ml). A set of 10 randomly selected clones for each recombinant was checked for Nef mRNA expression by RNA-PCR. Nef expression was verified by Western blotting of cell extracts after SDS-PAGE using INDIA-His probe (Pierce Chemicals, N.Y., USA) to detect 6-His-tagged Nefs. In almost all cases, all 10 randomly selected clones expressed Nef mRNA and protein. About 100–200 clones representing a statistical distribution of various chromosomal integrations for each recombinant were pooled and frozen as mass survivor cells.

For transcriptional studies, frozen cells were regenerated in medium devoid of G418. After 3-day passage, approximately 5.0×10^6 freshly regenerated cells were seeded on a 500-cm^2 square tissue culture dish containing 100 ml of DMEM without G418, and the cells were harvested when they were about 60% confluent (36–48 h).

RNA Isolation

Total RNA was isolated from 5.0×10^6–1.0×10^8 cells using the RNeasy Midi kit (Qiagen Inc., Calif., USA) as per the manufacturer's instructions, with slight modifications. Briefly, cells were lysed with RLT buffer and homogenized by sonication using the Virsonic 60 sonicator (The VirTris Company, N.Y., USA) with a power setting adjusted to intensity level 3 for approximately 3–5 min, until the cell lysate became nonviscous. After the final elution from the spin column with RNase-free water, the 300-µl elute was extracted with 1 ml of Trizol (Life Technologies Inc., Md., USA) and centrifuged at 4,000 *g* for 10 min at 25°C. RNA was precipitated from the aqueous phase with 500 µl of isopropanol. The pellet was washed twice with 75% ethanol, dried and resuspended in 200 µl of RNase-free water. The RNA was quantitated by UV spectrometry. The integrity of 28S and 18S fragments was confirmed by gel electrophoresis. The RNA was ethanol precipitated and stored at –80°C as a dispersed precipitate until further use.

Preparation of Microarray Slides

About 6,000 cellular genes were printed onto poly-*L*-lysine coated glass slides as described elsewhere [60, 77]. After the deposition of all the DNAs, the printed slides were washed to remove the salt and preblocked to prevent nonspecific binding of target, denatured and UV crosslinked [33].

Preparation of Probe and Hybridization

Fluorescently labeled cDNA probes were made from 200 µg of total RNA for Nef-expressing HeLa cells (Cy5-dUTP labeled) and 50 µg of total RNA for non-Nef-expressing HeLa cells (Cy3-dUTP labeled) by oligo(dT)-primed polymerization using SuperScript II Reverse Transcriptase (LTI Inc.) as described elsewhere [60, 77].

Scanning and Analysis

Scanning and analysis were performed essentially as previously described [23, 77]. Normalization across duplicate experiments was also done to compensate for array variability. The data obtained from the Nef-expressing and mutant Nef-expressing cells were tested for statistical significance ($p \leq 0.05$) using the SuperANOVA program for Macintosh platform.

Northern Analysis

Twenty micrograms of total RNA from each sample was electrophoresed in denaturing formaldehyde 1% agarose gels. Following overnight capillary transfer to Nytran plus membranes (Schleicher & Schuell Keene, N.H., USA), the blots were probed with PCR-amplified fragments of the selected genes of interest, which were radioactively labeled with a random-primed DNA labeling kit (Amersham Life Science Inc., Ill., USA). Hybridization was carried out for approximately 18 h in NorthernMax Prehybridization/Hybridization buffer (Ambion Inc., Tex., USA). The membrane was washed twice with 1 × SSC and 0.1% SDS for 15 min each and then washed twice with 0.25 × SSC and 0.1% SDS for 15 min each and autoradiographed. The image was scanned using a Phosphorimager (Molecular Dynamics Inc., Calif., USA) and analyzed by ImageQuant™ software from Molecular Dynamics.

Results

Effect of wt-Nef on the HeLa Cell Gene Expression Profile

In order to determine the effect of Nef on gene regulation, we utilized DNA microarray technology to obtain an overview of cellular genes exhibiting altered expression in the presence of Nef. HeLa cells were stably transfected with wt-Nef- and mutant Nef-expressing plasmids. Nef

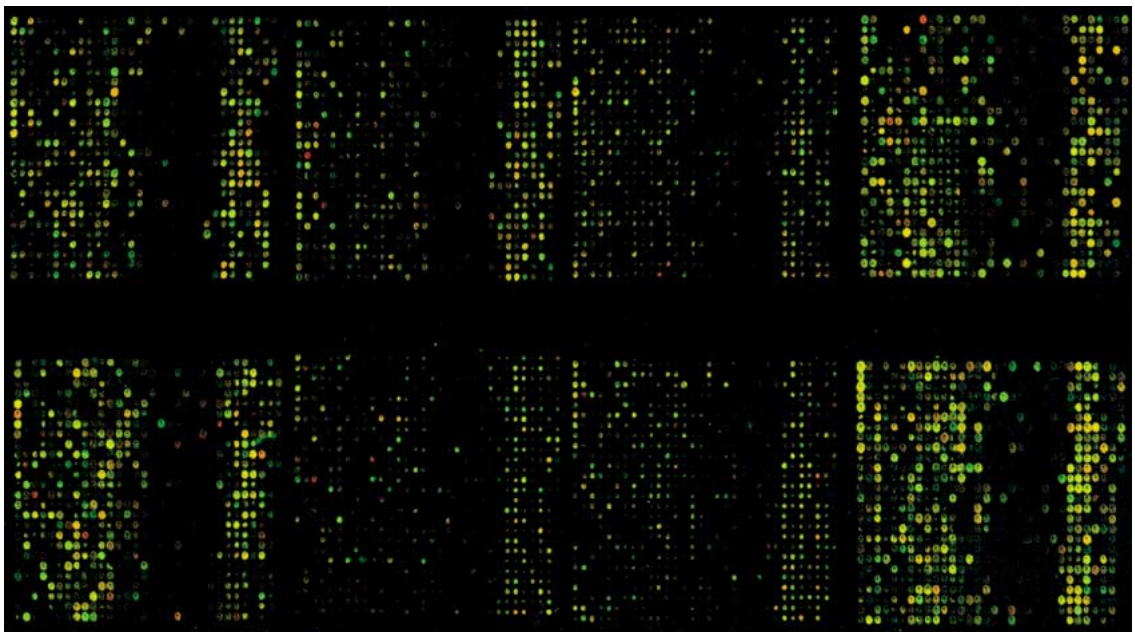

Fig. 2. An example of an array containing 6,000 cellular genes hybridized with fluorescently labeled cDNA synthesized using RNA frm HeLa cells and HeLa cells expressing HIV-1 NL4-3 Nef. The false color image indicates hybridization targets for genes upregulated in the presence of Nef in red and downregulated in the presence of Nef as green spots.

expression was verified by RT-PCR (not shown) and Western blot analysis (fig. 1). The levels of protein expression in HeLa-Nef and HeLa cells expressing Nef mutants were comparable, so that any differences in expression observed between wt-Nef- and mutant Nef-expressing cells were unlikely to be due to quantitative effects. To study gene expression profiles in the presence of Nef, RNA was initially isolated from Nef-expressing HeLa cells and control HeLa cells. The isolated RNA was used as the template in oligo-dT-primed reverse transcription reactions. These reactions were conducted in the presence of either Cy5- or Cy3-fluorescently labeled dUTP, producing appropriately labeled cDNA probes from the cells whose expression patterns were to be compared. Following probe purification, the fluorescently labeled cDNA probes were hybridized to an array consisting of about 6,500 elements containing PCR-amplified gene fragments from selected cellular genes. The arrays were scanned and fluorescence data were extracted at the Cy3 and Cy5 emission maxima. Data were compiled from the scan, and false color images were constructed to provide an initial qualitative sense of the differences in gene expression that characterize the cell lines being compared. Figure 2 shows one such false color image, in which spots representing genes overexpressed in the HeLa-Nef cells are shown in red, while genes underexpressed in the HeLa-Nef cells are shown as green spots. Genes that did not show altered expression are shown as yellow spots. Quantitative information was extracted from each spot, and quality control criteria, such as spot size and intensity above background, were applied prior to further analysis. A set of invariant housekeeping genes spotted on the arrays was used to produce a normalization factor (calibration factor) for each microarray experiment. An expression ratio for each gene was obtained by dividing the signal intensity obtained for HeLa-Nef cells by the signal intensity obtained for control HeLa cells. A calibrated expression ratio was obtained by multiplying the expression ratio for each gene by the calibration factor. Calibration ratios greater than 1 indicate that the gene is upregulated. A calibration ratio of less than 1 indicates downregulation. For our analysis, a cutoff value for the calibrated expression ratio of ≥ 2.5 was chosen to define an upregulated gene, while a calibrated expression ratio of ≤ 0.5 was used to define a downregulated gene. For spot intensity values, only genes with a minimum of 50 pixels in both the red and green channels were included. Based on these parameters, 137 genes were upregulated in the presence of Nef and 75 genes were downregulated in the presence of Nef. The calibrated expression ratios of the upregulated genes ranged

Table 1. Comparison of selected genes upregulated in HeLa-Nef cells and in HeLa cells expressing Nef mutants

Clone description		Calibrated expression ratios			Comments/functional significance
clone ID	clone name	Nef NL4–3	Nef MGG→MAA	Nef PXXP→AXXA	
31072	proprotein convertase subtilisin/kexin type 1	9.21	0.703	0.81	prohormone convertase, subtilisin serine protease family, cleaves proinsulin at B-chain/C-peptide junction, cleaves gp160 to gp120 + gp41
526657	transcription elongation factor B (SIII) (elongin A)	9.09	1.28	4.44	active subunit of elongin, suppresses transcriptional pausing
362853	protein tyrosine kinase; PTK9	7.22	1.10	0.71	widely expressed, conserved kinase, only distantly related to others, function unknown
358531	c-jun proto-oncogene (JUN)	6.75	1.26	0.99	transcription factor, AP-1 component
43129	topinsomerase I mRNA	6.14	1.10	3.24	transcriptional activator, relieves RNA pol-generated supercoils
44975	human homologue of yeast IPP isomerase	5.69	0.89	0.88	isopentenyl diphosphate:dimethylallyl diphosphate isomerase, first step in isoprenoid synthesis
363799	CLK-1 (CDC-like kinase)	5.36			homologies to cdc2, interacts and phosphorylates SR splicing factors extracellular matrix
196612	matrix metalloproteinase 12 (macrophage elastase)	5.35	1.09	0.82	degrades extracellular matrix, hypothesized to enhance HIV pathogenesis by promoting spread of virus through tissues, establishes reservoirs
292806	chromosome segregation gene homologue; CAS	4.78	1.09	3.24	mediates nuclear reexport of importin α
428248	activating transcription factor 3; ATF-3	4.54			ATF/CREB family member, full-length protein a suppressor, target of hepatitis B pX protein
346534	CDC2	4.39	2.48	2.00	catalytic subunit of M-phase promoting factor, overexpression would potentially alter cell cycle progression
162211	peroxisomal membrane protein 1 (Zellweger syndrome)	4.17	–	–	half-ATP-binding (ABC) transporter protein, transports long-chain FA-CoA to peroxisome for β-oxidation
898092	connective tissue growth factor (CTF, IGFBP8)	4.15	0.96	–	insulin-like growth factor binding protein family member, related to chicken nov proto-oncogene, mediates angioproliferation
51621	helicase-like transcription factor; HLTF-1	4.10	2.65	1.11	HTLF-1/HIP116, binds HIV LTR initiator element, potentiates activation by Sp1, upregulates HIV gene expression
295889	nuclear encoded mito serine hydroxymethyl transferase; SHMT	4.06	1.95	0.95	catalyzes transfer of methyl units, mitochondrial enzyme, greater involvement in glycine synthesis
273435	YES1 human homologue Yamanuchi sarcoma virus yes oncogene	4.06	2.72	1.80	src family nonreceptor protein tyrosine kinase
788493	TRAMP	4.02	3.72	2.84	mediates translocation of nascent proteins into ER, regulates translational pausing
28475	crystallin zeta (quinone reductase)	4.01	1.14	1.32	involved in metabolic pathways producing NADPH
471218	transcription factor AREB6	3.99	2.55	2.41	zinc finger transcription factor, regulates Na,K-ATPase, extended version of IL-2 negative regulatory factor
785793	human capping protein alpha subunit isoform 1 mRNA	3.99	1.21	1.56	regulates growth of actin filaments, affects cytoskeletal development
32790	homologue of mutS; hMSH2	3.98	1.69	0.96	DNA mismatch repair protein, mutations associated with hereditary nonpolyposis colon cancer
132911	protein phosphatase 1, catalytic subunit, beta isoform	3.95	0.78	0.76	controls terminal step of intracellular membrane fusion, required for protein transport between ER and golgi
897971	β-COP	3.76	1.14	1.70	mediates transport from ER to cis-Golgi, interacts physically with Nef to cause CD4 degradation in endosomes
74566	CRM1 protein	3.72	0.79	0.80	mediates nuclear export of proteins, also exports HIV-Rev and viral RRE-containing RNA
208718	annexin 1	3.44	0.90	1.30	inhibits phospholipase A2, thus inhibiting prostaglandins and leukotrienes, may blunt immune response to viral infection

The IMAGE consortium clone ID, the name of the gene and comments concerning the identity of the gene and/or its function are listed. The calibrated expression ratios for the cellular genes most highly overexpressed in the Nef-expressing HeLa cells (Nef NL4-3) compared to non-Nef-expressing HeLa cells are tabulated. Also listed are the calibrated expression ratios for the MGG→MAA myristylation site Nef mutant HeLa cells and the PXXP→AXXA SH3 mutant HeLa cells. Both mutations essentially abolish the upregulation seen in wt-Nef HeLa cells.

from 11.5 to 2.5. In this group are included all the genes which were upregulated to a statistically significant level ($p \leq 0.05$). We subsequently constructed a more refined list of differentially regulated genes which showed at least a 3-fold increase in expression in the presence of Nef (table 1). A discussion of the 25 genes most upregulated in the presence of Nef is given below. Nef effects on the transcriptional abundance of 12 members of this group were confirmed independently by RNA filter blotting analysis (fig. 3, table 2). Since the two methods employed, i.e. microarray and Northern blot analysis, have different technical characteristics, the results are not identical, but generally similar differences in expression were observed. Some of the upregulated genes have potential effects on HIV replication and pathogenesis. Some of these genes have also been previously identified as being significantly

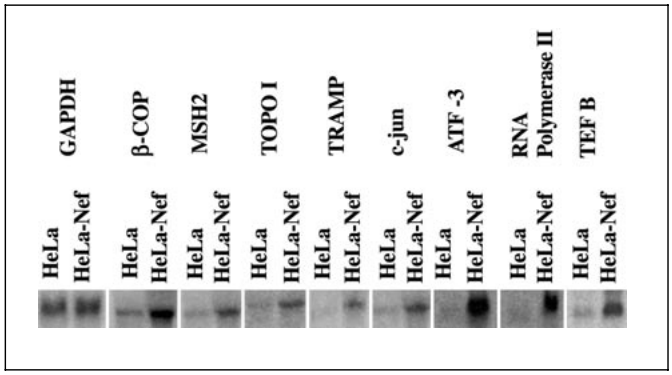

Fig. 3. RNA blot analysis of selected genes found to be upregulated in HeLa-Nef cells by microarray. Results from RNA blot analysis confirmed the upregulation of these genes. The amount of upregulation as determined by the microarrays was generally confirmed by the RNA blots (table 2). TEF B = Transcription elongation factor B.

Table 2. Comparison of calibrated expression ratios with observed RNA blot ratios for selected genes upregulated in Nef-HeLa cells

Clone ID	Calibrated expression ratio	Blot ratio	Clone name
526657	9.09	10.08	transcription elongation factor B (SIII) (elongin A)
44975	5.69	3.33	human homologue of yeast IPP isomerase
428248	4.54	5.51	activating transcription factor 3; ATF-3
346534	4.39	4.54	cdc2
51621	4.10	4.49	helicase-like transcription factor; HLTF-1
788493	4.02	4.63	TRAMP
471218	3.99	2.69	transcription factor AREB6
32790	3.98	2.39	homologue of *E. coli* mutS; hMSH2
897971	3.92	2.78	β-COP
813410	3.27	4.26	human RNA polymerase II subunit
208718	3.44	2.01	annexin I
35516	3.15	2.33	cyclin G1

The calibrated expression ratio and the expression ratio obtained from the RNA blots for several selected genes are compared. The degree of upregulation as determined by microarray (calibrated expression ratio) generally agrees with the degree of upregulation as determined by RNA blot.

involved in HIV infection. Some cellular genes were also downregulated in the presence of Nef (table 3). A few of the genes identified in this study provide information on other possible cellular pathways within host cells that may be modulated by Nef.

Significance of Genes Upregulated in HeLa-Nef Cells

Following is a brief description of the top 25 genes that were upregulated in the presence of Nef but not by the mutant Nefs. The genes are categorized based on their known or probable cellular function. A number of the proteins encoded by these genes have been shown to either interact with Nef (e.g. β-COP) using functional studies, or are otherwise involved in HIV pathogenesis, indicating an attempt by the virus to regulate the host cell cycle at an early stage of infection.

Table 3. Genes downregulated by Nef-HeLa and Nef-HeLa mutants

Clone description		Calibrated expression ratios			Comments/functional significance
clone ID	name	Nef NL4-3	Nef MGG→ MAA	Nef PXXP→ AXXA	
742082	phosphoenolpyruvate carboxykinase 1 (soluble)	0.14	1.14	0.53	involved in combating oxidative stress
774409	endoglin (Osler-Rendu-Weber syndrome 1)	0.22	0.77	0.76	may modulate TGF-β signaling, thereby involved in angiogenesis
275738	carbonic anhydrase I	0.25	1.14	1.38	involved in CO_2 and lactic acid elimination
43743	*Homo sapiens* delta-catenin	0.29	0.65	0.76	expressed in nervous system, may be involved in tissue morphogenesis and cell motility

The calibrated expression ratio for four genes found to be downregulated in the wt-Nef-expressing HeLa cells (nef NL4-3) are listed. The calibrated expression ratios observed for these genes in the MGG→MAA myristylation mutant and the PXXP→AXXA SH3 mutant are also listed. The mutants generally abolished the downregulation observed with wt-Nef.

Viral Infection and Pathogenesis

Protein convertase subtilisin/kexin type 1 (PCSK1, PC1, PC3; from IMAGE clone ID 31072) is a prohormone and protein convertase from the kexin/subtilisin family of serine proteases, expressed principally in neuroendocrine cells [79]. PC1, as well as other members of the kexin/subtilisin family, has been shown to cleave gp160 into gp120 and gp41 [31, 32, 101], a key step in the HIV viral life cycle. This step is required for an infectious virus [74]. A *nef*-mediated upregulation of PC1 could lead to an increased efficiency in processing gp160 to gp120 and gp41, thereby facilitating viral infectivity. PC1 also cleaves proinsulin at the B-chain/C-peptide junction, initiating proinsulin processing. PC1 appears to play an important part in the regulation of glucose homeostasis because glucose coordinately regulates insulin and PC1 expression. Some evidence suggests that alterations in glucose metabolism may accompany viral infections generally and HIV infection specifically, perhaps to supply the increased metabolic demands that may result from viral infection [96].

Matrix metalloprotease 12 (MMP12, macrophage elastase, human macrophage elastase, HME; from IMAGE consortium clone 196612) [92] is one of a family of enzymes that can degrade the extracellular matrix and are important in tissue remodeling and repair, tumor metastasis and inflammatory processes. MMP12 is expressed in macrophages and in placenta and can be induced by agents such as lipopolysaccharide [12]. Interestingly, HIV infection has previously been shown to stimulate the expression of another matrix proteinase, the 92-kD type IV collagenase [106], and the increased expression of matrix metalloproteinases during HIV infection has been hypothesized to contribute to viral pathogenesis by enhancing the spread of the virus through tissues, resulting in the establishment of tissue viral reservoirs [105].

Annexin I (ANX I, lipocortin I, LPC I, calpactin II) [102] is one of a family of proteins that inhibit phospholipase A_2. The inhibition of phospholipase A_2 inhibits the production of arachidonic acid, thus inhibiting the production of a variety of inflammatory mediators, prostaglandins and leukotrienes. ANX I binds to the cytosolic face of the plasma membrane in a Ca^{2+}-dependent fashion. ANX I has demonstrated anti-inflammatory activity [26]. It also has other effects, including cell proliferation, differentiation and neutrophil migration [39, 62, 81]. The anti-inflammatory activities of ANX I might help blunt an immune response directed at the HIV-infected host cell, although this is somewhat counterintuitive, since factors that stimulate host cells enhance viral replication.

Transcriptional Regulation

Human topoisomerase I (TOP1; from IMAGE consortium clone 43129) [30, 57], a type 1 DNA topoisomerase, interconverts DNA topologic forms through transient single-strand breaks [reviewed in ref. 47, 103]. TOP1 probably functions principally during transcription, relieving supercoils generated by the passage of RNA polymerase; it is an important transcriptional activator, increasing the formation of active TFIID-TFIIA complexes [94]. TOP1

expression can change substantially, regulated by a variety of treatments and activators and mediated at least in part by Nuclear Factor-Interleukin-6 (NF-IL6) [52].

Transcription elongation factor B (TCEB3, elongin A) [5–7] is the transcriptionally active 110-kD subunit of the elongin RNA pol transcription factor which suppresses pausing during transcription, thus increasing elongation. Elongin A appears to stimulate elongation after 8 or 9 nucleotides have been transcribed, in the presence of RNA polymerase II, TBP and TFIIB. The upregulation of elongin A by Nef may be particularly interesting given the similar transcriptional antitermination effect of the HIV Tat transacting protein.

Activating transcription factor 3 (ATF3; from IMAGE consortium clone 428248) [49] is a member of the activating transcription factor/cAMP-responsive element ATF/CREB family of transcription factors. Its binding site exists in several viral and cellular genes, including adenovirus E1A-inducible viral genes and cellular cAMP-inducible genes. The factor exists in two alternatively spliced forms. The full-length form contains a leucine zipper domain and binds DNA. It represses rather than activates transcription, perhaps by stabilizing the interaction of inhibitory cofactors with the promoter. ATF appears to be the target of another viral protein, the hepatitis B virus pX protein, which binds to ATF3 and increases its ability to repress transcription [9]. ATF3 is also upregulated during the exposure of cells to ionizing radiation [2].

Helicase-like transcription factor (HTLF-1, also known as HIP116, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin) has a DNA binding domain, a RING finger domain, seven DNA helicase domains and DNA-binding and DNA-dependent ATPase activities. HTLF-1 appears also to interact directly with Sp1 and potentiates transcriptional activation by Sp1 [34]. Given the apparent potential involvement of HTLF-1 in the regulation of HIV gene expression and the ability of HTLF-1 to work together with Sp1 to increase transcription, HTLF-1 upregulation may enhance transcription from the HIV Long terminal repeat (LTR).

AREB6, a zinc finger protein isolated from a HeLa cell library [104], can affect transcription from the ATPase promoter. AREB6 appears to represent an extended version of another zinc finger transcription factor (TCF8, Nil-2-a, T-lymphocyte-specific IL-2 inhibitor) [108] originally cloned as a negative regulator of IL-2 expression. The Nef expression phenotype may then be thought of in terms of abatement of the autocrine loop of T cell activation.

Nuclear Important and Export

CDC28/CDC2-like kinase (CLK1; from IMAGE consortium clone 363799) [56] is a human protein kinase with homologies to the yeast CDC28/CDC2 kinases that regulate the cell cycle. The mouse homologue can interact with serine-arginine-rich (SR) proteins in vivo and phosphorylate them in vitro [29]. SR protein phosphorylation presumably leads to alterations in SR protein function with alterations in splice site utilization. Since the regulation of HIV gene expression depends on the pattern of splicing modulated by Rev, alterations in SR protein activity might serve as a secondary mechanism to modulate HIV RNA splice site utilization and gene expression, particularly since the expression of certain SR proteins appears to change during HIV infection [71].

CRM1 (exportin 1; from IMAGE clone 74566) mediates the nuclear export of proteins containing a leucine-rich nuclear export signal [40, 65]. CRM1 is intimately involved in critical processes of HIV replication. CRM1 mediates the nuclear export of the HIV transacting protein Rev and viral Rev-responsive element (RRE)-containing RNA, allowing for the nuclear export of singly spliced and full-length viral messenger and genomic RNA. The overexpression of CRM1 leads to increased nuclear export of Rev [40]. Augmented CRM1 expression and the effects on the importin α pathway may result in enhanced nuclear shuttling that may be beneficial to HIV replication in nondividing cells.

Protein Transport and Translocation

The translocation chain-associated membrane protein (TRAMP; from IMAGE clone 788493), originally identified in the dog [44], is an important component of the machinery responsible for translocating nascent proteins into the endoplasmic reticulum (ER). TRAMP helps mediate the interaction of the nascent protein with the translocational machinery and regulates translocational pausing. Thus, it ensures that proteins entering the ER are optimally exposed to the cytosol, thereby presumably regulating posttranslational processing, modification and subsequent degradation [51]. Upregulation of TRAMP may have the salutary effect of hastening the maturation and transport of viral glycoproteins in addition to reducing the levels of misfolded proteins that may be retrieved by proteosomes and presented as 'foreign' peptides in the context of the MHC-I receptor.

β-COP is a 110-kD protein that comprises part of the coat of non-clathrin-coated vesicles. It exists in both a cytosolic complex and a membrane-bound form [36] and mediates transport of protein from the ER to the cis-Golgi

[82]. Nef has been found to physically interact with β-COP [13]; it targets CD4 for degradation by serving as a connector between CD4 and β-COP in endosomes [85]. The upregulation of β-COP by Nef may be due either to specific effects of Nef on β-COP expression or through secondary effects such as the perturbation of β-COP homeostatic feedback control mechanisms resulting from the loss of β-COP as a result of sequestration by Nef. In any case, the upregulation of β-COP associated with Nef expression may serve to potentiate the activities of Nef that result from interactions with β-COP, such as CD4 downregulation.

The YES proto-oncogene (YES1; from IMAGE clones 273435 and 133178) is homologous to the Yamaguchi sarcoma virus yes oncogene [91, 99]. It is a relatively less well-characterized src family nonreceptor protein tyrosine kinase.

Protein phosphatase 1 [8, 87] (PP1; from IMAGE clone 132911) is a serine/threonine-specific protein phosphatase that controls the terminal step of intracellular membrane fusion [83]. PP1 functions at a relatively late stage of vacuolar fusion, probably at the step of membrane mixing. Within the cell, PP1 appears to be required for the transport of proteins from the ER to the Golgi because inactivation of PP1 blocks the transport of a test protein, carboxypeptidase Y, from the ER to the Golgi [83].

Protein tyrosine kinase 9 (PTK9) [11] is a 350-amino acid protein cloned from an embryonic lung fibroblast library with antiphosphotyrosine antibodies. It is expressed in many tissues and is evolutionarily conserved, but is unrelated to other protein kinases. The bacterially expressed protein phosphorylates several substrates, but its physiologic function remains unknown.

Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IPP isomerase; IMAGE consortium clone ID 44975) [48, 109] catalyzes the isomerization of IPP to dimethylallyl pyrophosphate, the essential first step in the production of long-chain isoprenoids, including iso-prenoids involved in protein prenylation. Reversible iso-prenylation of proteins facilitates protein-protein interactions and membrane-associated protein trafficking. The loss of proper localization of Ras and other small Ras-like G proteins when their prenylation is inhibited has permitted a new target for antineoplastic modalities. Upregulation of IPP levels by Nef may be expected to modulate intracellular signaling pathways involving Ras and Ras-like proteins and affect cellular protein trafficking.

Actin capping protein α [21] (CAPZA1; from IMAGE clone 785793) was originally identified in chicken as one subunit of a heterodimeric barbed-end actin-binding protein that regulates the growth of actin filaments at the barbed end [20, 22]. These activities may be involved in the rearrangement of the cytoskeleton and may be recruited by HIV for the delivery of preintegration complexes into the nondividing nucleus and may facilitate the transport of nascent HIV nucleocapsids.

Cellular Metabolism

Peroxisomal membrane protein 1 (PXMP1, peroxisomal membrane protein 70 kD, PMP70; from IMAGE consortium clone 162211) is one of the two major proteins of the peroxisomal membrane. It is one of the half-ATP-binding cassette (ABC) transporter proteins. PXMP1 serves to transport long-chain acyl-CoA across the peroxisome membrane for subsequent β-oxidation [55].

Mitochondrial serine hydroxymethyltransferase (SHMT2; from IMAGE clone 295889) [98] catalyzes the transfer of methyl units from the serine donor to 1-carbon-substituted cofactors in a wide variety of important metabolic processes, including purine, thymidine, methionine and glycine biosynthesis. There are both cytosolic and mitochondrial isoforms of the enzyme. The precise roles of the isozymes are not clear, although the mitochondrial enzyme may have a greater role in glycine biosynthesis [76, 84]. Glycine also serves as the source of methyl groups in many biosynthetic processes, including nucleic acid biosynthesis.

Crystallin zeta or quinone oxidoreductase [43] (CRYZ; from IMAGE clone 28475) is a protein that is not related to the other lens crystallins. It is expressed at high levels in a variety of nonocular tissues. The implications of the overexpression of this gene during HIV infection are not clear. However, viral infection likely places substantial metabolic and biosynthetic demands upon the host cell, so an additional source of NADPH might increase the efficiency of host cell biosynthetic pathways.

Cellular Proliferation and Apoptosis

The human c-jun proto-oncogene [3, 15, 50] (from IMAGE consortium clone ID 358531) [reviewed in ref. 4, 107] is a bZip protein which forms homodimeric complexes or heterodimeric complexes with other bZip proteins from the *fos* and CREB families. It contributes to AP-1 transcription factor complexes and binds specific promoter sequences. c-jun helps regulate cellular differentiation (including monocyte differentiation), proliferation, apoptosis and stress responses, so the differential expression of c-jun might be expected to have significant effects on cells supporting HIV replication.

The human homologue of the yeast chromosome segregation gene CSE1, also known as the 'cellular apoptosis susceptibility' gene (CAS; from IMAGE clone 292806) [reviewed in ref. 17] [18], was originally cloned as a gene, the antisense version of which rendered breast cancer cells resistant to immunotoxins and tumor necrosis factors α and β. The gene was found to have increased expression during cell proliferation, in actively dividing cells [19] and in some tumor cell lines. CAS mediates the reexport from the nucleus of the nuclear import receptor importin, a component of the importin nuclear import pathway for Nuclear Localization Signal-containing protein. CAS acts as part of an importin α/CAS/RanGTP complex [66]. Although the activities of the cellular nuclear import pathways appear to be intimately linked with the HIV replication process, the nuclear import of Tat and Rev appear to depend on interactions with importin β, not importin α [53, 100]. Upregulation of importin α may facilitate the recruitment of alternative nuclear importation pathways for Rev, Tat and preintegration complexes.

CDC2 is the human homologue of the fission yeast cdc2 (budding yeast CDC28) gene [68]. It is the catalytic subunit of M-phase promoting factor, the protein kinase complex that controls entry into mitosis and constitutes a critical checkpoint in the cell division cycle. Overexpression of CDC2 is likely to have significant effects on the cell cycle.

Connective tissue growth factor (CTGF, insulin-like growth factor binding protein 8, IGFBP8; from IMAGE clone 898092) [16, 61], distantly related to platelet-derived growth factor, is a member of the insulin-like growth factor binding protein family and is closely related to the chicken nov proto-oncogene [73]. A variety of stimuli lead to its induction. CTGF appears to regulate a variety of proliferative processes, including fibrotic and atherosclerotic diseases. Most interestingly from the point of view of HIV pathogenesis, it appears to mediate certain angioproliferative responses [93].

MSH2 (FCC1, COCA1, HNPCC1, homologue of *Escherichia coli* mutS) [67] is a DNA mismatch repair protein. Although it is not immediately clear how the overexpression of such a gene might contribute to HIV replication, the generation of a retroviral provirus is likely to produce DNA mismatches [63] which may trigger host cell apoptosis if not efficiently repaired.

Effect of Mutant Nefs on the HeLa Cell Gene Expression Profile

Studies have shown that myristylation of glycine at position 2 and the PXXP domain of Nef are important for the function of Nef. Lack of myristylation and/or the PXXP motif inhibits Nef from effectively interacting with cell signal transduction molecules. In order to study the effects of these Nef domains on transcriptional regulation, we constructed a mutant that lacked the myristylation motif and another that lacked the PXXP motif. We produced HeLa cells expressing these mutants and examined the effect of the expression of the mutant Nefs on the expression profiles of the same set of cellular genes. These mutant Nef studies indicate that certain structural determinants in Nef are critical for its effects on cellular gene regulation. The myristylation mutant MGG→MAA and the SH3 domain mutant PXXP→AXXA both lost their ability to upregulate most of the genes that were upregulated by wt-Nef (table 1). Genes that were downregulated by Nef were not affected by the presence of either of the mutants (table 3). While both mutants generally behaved in a similar fashion, some genes were differentially affected. For instance, the PXXP mutant but not the myristylation mutant had an effect on TOP1, similar to wt-Nef. In contrast, upregulation of CDC2 (table 4) was not observed when the PXXP mutant was expressed. We also observed that certain genes were upregulated in the presence of the mutant Nefs, but not by wt-Nef (table 4). This finding suggests that lack of a myristylation and/or PXXP domain may mistarget Nef, so that Nef interacts with proteins other than those with which it usually interacts, resulting in the increase in expression of genes not ordinarily targeted by Nef. In any large-scale survey of the global changes in gene expression that accompany a particular treatment or event, the effects observed may result from the direct effect of the treatment, from indirect effects of the treatment or from attempts by the host cell to compensate for other effects caused by the treatment. The present experiments are not sufficient to determine which of these mechanisms is responsible for the observed changes in cellular gene expression. However, the observation that some of the genes affected by Nef are known to be involved in HIV replication suggests that the Nef-mediated alteration in cellular gene expression is likely to have pathogenetic significance regardless of the mechanisms responsible for the alterations in expression.

Discussion

Our results show that Nef expression in HeLa cells leads to the differential expression of a restricted subset of cellular RNAs from an overall set of over 6,000 genes. These alterations in cellular gene expression are likely to

Table 4. Genes selectively regulated in either MGG→MAA or PXXP→AXXA mutants

Clone description		Calibrated expression ratios				
clone ID	name	Nef NL4-3	up in Nef MGG→ MAA only	up in Nef PXXP→ AXXA only	up in Nef mutants only	
					MGG→ MAA	PXXP→ AXXA
41452	spermidine/spermine N-acetyltransferase	3.02	1.99	2.26	–	–
428248	activating transcription factor 3	3.90	1.73	2.09	–	–
43129	topoisomerase I mRNA	6.14	1.10	3.24	–	–
898286	cell division cycle 2, G1 to S and G2 to M	3.77	2.45	1.36	–	–
40017	H+ ATP synthase subunit B	1.35	–	–	2.57	2.81
49608	cytochrome c-1	1.38	–	–	2.72	2.73

The calibrated expression ratios for several genes found to be differentially regulated in one or another Nef mutant are listed. Nef NL4-2 = wt-Nef; MGG→MAA = Nef mutated in the myristylation site; PXXP→AXXA = Nef mutated in the SH3 domain.

be directly and specifically related to the expression of Nef, because the expression profile of HeLa cell genes expressing wt-Nef was notably different to the expression profiles of cells expressing Nef mutated in two key regions. These mutations affected the myristylation site that leads to the targeting of the protein to the interior face of the host cell plasma membrane and the PXXP motifs that mediate the interaction of Nef with cellular kinases leading to activation of cellular signal transduction pathways.

While certain genes (distinct from those seen in wt-Nef-expressing cells) were upregulated in the PXXP→AXXA Nef mutant cells, this effect was not observed in cells expressing the myristylation (MGG→MAA) mutant. Since neither mutant induced upregulation of transcripts that were affected by wt-Nef, N-terminal myristylation and the PXXP domain appear to be crucial for the effects of Nef on transcriptional regulation. Also, expression levels of some genes were reduced in the presence of Nef, suggesting that the set of upregulated genes does not reflect overall nonspecific activation of cellular physiology. Instead, the altered expression may reflect gene expression changes due to subtle changes in cellular activation pathways. Recent results indicating that mutations in Nef that result in aberrant subcellular localization also destroy the ability of Nef to enhance virion infectivity in ways that are independent of some of its other effects (e.g. effects on viral protein processing) reinforce the hypothesis that misdirected Nef can fail to interact with other viral and host cell factors needed to mediate the effects of Nef or can interact with other factors so as to block the usual effects of Nef on viral replication [37].

Nef has been demonstrated to alter host cell physiology at a posttranscriptional level through direct interactions with cellular gene products such as CD4, MHC-I, p21-activated kinase, adaptins and β-COP. At least some of the differentially expressed genes are likely to enhance viral replication. Since Nef is expressed early during the viral replication cycle, certain activities of Nef may be required during the initial stages of viral gene expression or may continue to be required for subsequent stages of viral replication. Certain genes affected by Nef, for example the nuclear import/export pathways, the membrane/vesicle fusion pathways and the transcription factors, may be overexpressed early in the life cycle. Alternatively, genes involved in cell cycle regulation or DNA mismatch repair may be expected to principally exert their effects on viral replication prior to HIV proviral integration and gene expression. If the upregulation of such proteins via Nef expression has evolved to enhance viral replication, it may be instructive to investigate whether these proteins are incorporated into virions.

Given the intrinsic potential of Nef to interfere with many cellular activation pathways, it is likely that the ultimate phenotype may be determined by the cell type and the activation status. Although HeLa cells can support HIV replication when engineered to express CD4, the effects of Nef on other cell types, including lymphoid and monocytoid cells, may differ.

Many of the Nef-induced changes in gene expression, such as the upregulation of cellular transcription factors, DNA repair mechanisms, nuclear export pathways, protein processing enzymes, factors affecting cellular metabolism and potential pathogenesis factors, could potentially serve to enhance the ability of the host cell to support HIV replication. Finding cellular genes already known to influence HIV replication and pathogenesis, e.g. HTLF-1, CRM1, β-COP and MMP12, among the upregulated cellular genes provides additional support for this hypothesis, suggesting that at least some of the other differentially expressed genes identified in this study may have pathogenic significance. Since Nef is dispensable for replication in tissue culture, the upregulation of these genes, while certainly not required for viral replication, may act to enhance viral pathogenesis. While this article was under review, another account of the effects of Nef on cells using gene expression profiling was reported. This account examined the effects of a tetracycline-inducible Nef on gene expression in Jurkat cells using radiolabeled cDNAs hybridized to membrane-immobilized probes [95]. Although the cell lines and technologies used in the two expression profiling studies had significant differences, the general conclusions of the two studies, i.e. that the early HIV gene Nef apparently acts to optimize the conditions for viral replication present in the host, are essentially similar. In several cases, the two studies both found that similar types of genes (i.e. transcription factors) were differentially regulated, including members of the same family of transcription factors, for example different members of the CREB/ATF family or components of the AP-1 transcription factor complex. Both studies found that some of the same genes were upregulated to close to the same degree, for example β-COP. There were a number of differences in the genes found to be upregulated, presumably due to the difference in viral isolate and cell types, differences in induction (constant expression vs. inducible expression), technical differences in array production, labeling and analysis, and the number and particular identity of the genes examined. For example, although HeLa cells can support HIV replication if they express receptors that HIV can use for entry [70], it is unreasonable to expect that they can exhibit the full range of phenomena associated with lymphocyte activation. Nevertheless, it may be informative to note that the expression of Nef from two different viruses is associated with changes in the gene expression profile of two distinct cell types and that the changes in the expression profiles of both these different cell types may be interpreted as making the host cell better suited to supporting HIV replication. Nef may therefore serve a similar role in this regard in different cell types, although mechanistic details may differ somewhat from cell type to cell type. It is important to note, however, that not all genes that are differentially expressed may be directly affected by Nef, and some of the alterations in expression may result from the effects of cellular feedback mechanisms that follow when Nef perturbs the expression of other genes. Further analysis of the effects of Nef on the host cell and the structural features required for Nef to alter host cell gene expression should offer additional insights into HIV pathogenesis and may suggest additional approaches to antiviral therapy.

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